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Biochimica et Biophysica Acta 1762 (2006) 973–985

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

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Review

Amyotrophic lateral sclerosis as a complex genetic disease

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Received 14 May 2006; received in revised form 25 July 2006; accepted 2 August 2006
Available online 5 August 2006

Abstract

In complex diseases like ALS, there are multiple genetic and environmental factors all contributing to disease liability. The genetic factors causing susceptibility to developing ALS can be considered a spectrum from single genes with large effect sizes causing classical Mendelian ALS, to genes of smaller effect, producing apparently sporadic disease. We examine the statistical genetic principles that underpin this model and review what is known about ALS as a disease with complex genetics.

Keywords: Complex disease; Complex genetic; Amyotrophic lateral sclerosis; Sporadic; Association study; Gene; Genetic

1. Introduction

1.1. What is a complex disease?

Most cases of amyotrophic lateral sclerosis (ALS) are isolated incidents. When, in 1955, Kurland and Mulder first provided evidence that in about 10% of cases there was a family history [1], the natural assumption was that the remaining sporadic cases did not have a genetic cause. This has to be viewed on the historical background of the time, when the idea that ALS might have any genetic component was surprising, ideas of Mendelian inheritance of disease were mainstream, and there was no adequate genetic model for non-Mendelian familial clustering or sporadic disease. We now have a more detailed and sophisticated understanding of the behaviour of genes, and it is reasonable to assume that in most if not all cases, there is a genetic contribution.

The discovery of mutations in SOD1 in 20% of familial cases [2,3] and between 2 and 7% of sporadic cases [4–8], indicates that at least some sporadic cases of ALS are genetic. Further evidence comes from a UK twin study, which examined mono- and dizygotic twins for concordance, having first eliminated probands from families already identified with dominant ALS inheritance. Heritability was defined as $h^2 = 2 \times [r_{mz} - r_{dz}]$, where r_{mz} and r_{dz} are the liability correlations for mono- and dizygotic twins ranging from 0 (fully non-genetic) to 1 (fully heritable). Based on these calculations, the heritability of ALS was shown in this study to be between 38 and 85% [9]. The authors concluded that "…the finding that between 38% and 85% of variation in (ALS) is due to inherited factors indicates that even in so-called sporadic (ALS), with a conservative approach to analysis of the data, genetic influences are significant."

Another way to examine the role genetic factors might play in sporadic ALS is to estimate the relative risk of ALS to siblings of affected individuals as compared with the population risk, given the symbol λ_s. Without detailed population risk data for ALS from genetic epidemiology, only imperfect guesses are possible. If it is assumed that since approximately 10% of cases have a family history, then the risk to a sibling of a sporadic case with an unknown family history is the risk of having a family history of ALS × the risk of sharing a dominant allele = 1/10 × 1/2 = 1/20. The lifetime risk of developing ALS estimated from population data and death certificate data as between 1 in 400 and 1 in 1000 [8]. Therefore, a rough estimate of λ_s is between (1/20)/(1/400) = 20 and (1/20)/(1/1000) = 50. The problem with this approach is that it assumes dominant inheritance, but we know from many pedigrees that familial clustering may occur without obvious Mendelian patterns, and these are excluded from this calculation. Nevertheless, this value for λ_s does provide some information.

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0925-4439/$ - see front matter © 2006 Published by Elsevier B.V.
doi:10.1016/j.bbadis.2006.08.001

Current thinking on complex diseases like ALS is that there are multiple genetic and environmental factors all contributing to disease liability. This arises as a direct result of modern genetic statistical theory, which itself is based on an extended version of Mendelian genetics. In Mendel's description of genetic transmission of heritable characters, each "formative element" was responsible for a single character, and each parent had two formative elements for each character, transmitting one randomly to the next generation in gametes. He showed the transmission of formative elements for different characters was independent, and that some character traits were dominant to others, so that the recessive trait was masked in one generation if the parent had both an element for a dominant and recessive character simultaneously. When Mendel's ideas were rediscovered at the turn of the 20th century, the formative elements were renamed genes, the characters phenotypes, and it was discovered that one of his laws, that of independent transmission of elements for different characters, was not always true. Some phenotypes were linked together in "linkage groups", in which transmission of the gene for one phenotype affected the probability of transmission of a different gene for another phenotype. We now know the physical basis of linkage groups is the arrangement of genes in chromosomes, and the distortion by linkage of transmission frequencies of different characters from the expected 50:50 is used as a means of disease gene mapping.

Mendelian traits are well recognised, but it is difficult to explain continuous phenotypes, such as height, on the basis of single discrete genes. Height has a strong inherited component, and can be predicted reasonably accurately from parental heights. A particle theory of genetics needs to be able to explain this continuous trait without the discontinuities implied by discrete genes, but there is no such obstacle for a wave theory of genetics in which waves transmit the genetic information through generations. There was thus a battle in the early years of genetics between those who considered genes to be particles, and those who considered them to be waves. This is not as ridiculous as it may sound today, as it was thought to be a logical explanation for the transmission of continuous characters, and was a view held even by William Bateson, the first professor of genetics in history (who is also responsible for coining the word "genetics"). The solution came in 1918 when Fisher, who founded much of modern statistics, showed that three or more genes each contributing to a trait, but each following Mendel's laws, would result in a phenotypic distribution indistinguishable from a normal distribution, particularly if there was also an environmental contribution. This is because the alleles for each gene occur in pairs in each individual, and the phenotypic traits therefore follow the binomial distribution. The binomial distribution approximates the normal distribution as n increases, and with $n>2$, the approximation is quite good. (For example, three loci with equally frequent alleles A and a, B and b, and C and c, in which capital alleles increase a trait, say IQ, by 5 and small case alleles decrease the trait by 5, result in IQs of 70, 80, 90, 100, 110, 120 and 130 with frequencies of 1:6:15:20:15:6:1). It was therefore possible to explain Mendelian traits and continuous traits using straightforward statistics applied to Mendel's laws.

Unfortunately, this approach still does not explain discrete traits showing familial clustering but without Mendelian inheritance. This was modelled successfully in 1981 with the so-called threshold liability model [10]. In this explanation, the liability to disease rather than the phenotype itself, is what is normally distributed (as in the three genes or more model), but disease only occurs once a critical threshold of liability is crossed. If the cumulative disease susceptibility burden is sufficient then the individual develops disease. This is classically illustrated with cleft lip and palate, which shows familial clustering but is not Mendelian. The "hidden" normally distributed phenotype, liability to cleft lip and palate, is in fact the speed and timing of fusion of the palatal plates. The exact speed at which they come together is unimportant so long as they meet before a critical developmental stage. This model transforms a continuous trait back into a binary phenotype/no phenotype discrete trait. The result predicted is familial clustering, but because of smaller family sizes and poor knowledge of extended families, cases can appear sporadic.

We now therefore have three models explaining three modes of genetic disease transmission: Mendelian—where discrete traits are inherited in a predictable fashion, the polygenic model for continuous traits, and the liability threshold model for a discrete trait with familial clustering which is not inherited in a Mendelian fashion. Other factors increasing the complexity of genetic predisposition to disease of which we are currently aware are: the multiple effects of single genes (pleiotropy), the interaction of multiple genes with each other (epistasis), the interaction of genes with environmental factors, splice variants of genes, variations in copy number, and post translational protein modification, but there will undoubtedly be others discovered in the future.

There is therefore complexity from the number and effect size of genes contributing to disease risk. There is a further layer of complexity at the level of phenotypic expression because the expression of one gene can alter the expression of another at transcription, at the level of molecular cell machinery, or based on the cell type, organ or system it is expressed in, and so a phenotype is rarely the product of a single gene, even if it seems so at first. Modifying genes may enhance or reduce a phenotype, produce novel phenotypes, or mask the effect of a disease gene, to produce an organism that resembles the wild-type. In Mendelian diseases, this may be manifested as reduced penetrance, for example, age or sex-dependent penetrance. Even when environmental factors are the same, traits which are inherited in a dominant manner on one genetic background can display recessive or co-dominant inheritance on another. Many other modifier effects are recognised, but all are examples of gene–gene or multiple gene–environment interactions. The "one gene, one trait" model of disease can therefore be regarded as too simple for any complete description of disease risk, even though it is useful for identifying genes for Mendelian diseases.

2. ALS susceptibility

The liability threshold model is now generally accepted as the best description and explanation of the behaviour of

complex diseases. Susceptibility to developing ALS can therefore be considered a spectrum from single genes with large effect sizes causing classical Mendelian ALS, to multiple genes of smaller effect, producing apparently sporadic disease. Those genetic causes of ALS so far identified and robustly replicated have all been in genes causing disease inherited in a classical Mendelian fashion, giving the appearance that all genetic ALS is familial and Mendelian. This is to some extent a self-fulfilling prophecy, since the gene-hunting techniques available at first required large pedigrees with clear inheritance patterns. What is now required is an approach to find the small effect genes that contribute together with each other and environmental factors to cause ALS. Hunting for such genes is possible using case-control association studies, but in the past these have been hampered by the large numbers of genetic markers and samples required to attain sufficient power. Until recently therefore, most studies have confined themselves to examining candidate genes selected because of structure, function or similarity to known genes. The problem is that in ALS little is known about the disease mechanism, making candidate gene selection difficult, haphazard, and the biased choice of a researcher. As a result, many studies performed to date have not found association, and only a few of those that have found association have been replicated.

For diseases such as ALS, with late onset and short survival, association mapping of disease genes in unrelated individuals is an important approach because obtaining the number of cases required for sufficient power in family-based studies is problematic. Genome-wide association studies, which use a strategy similar to linkage scans, uses large numbers of informative markers to identify association between allelic variants and disease. This is the ideal approach for diseases like ALS where the etiology remains largely unknown as it makes no *a priori* assumptions about the location of the variants of interest. However, the extent of linkage disequilibrium in unrelated samples is small and this means that very dense marker sets must be used on large sample sizes in order to generate sufficient power to detect association. This has meant that the costs and workload involved in such studies was unfeasible. To get around this problem, most studies have chosen a candidate gene approach where candidates are chosen based on hypotheses of disease causation. Unfortunately, such studies have so far been inconsistent and frequently underpowered. Here we review some of the most salient studies to date.

2.1. Apolipoprotein E

The difficulties in unravelling the genetic architecture of sporadic ALS are well illustrated by Apolipoprotein E (APOE) which has been studied both as a risk factor for ALS and as a modifier of various phenotypic aspects. APOE is one of the molecules responsible for the chaperoning of cholesterol through the bloodstream. It is polymorphic in the general population, with two SNPs forming three major haplotypes named as alleles, E2, E3 and E4 (the fourth, E1, does not tend to occur) [11]. Genetic variants of APOE are implicated in many abnormalities of blood lipids and cardiovascular disease, as might be expected, but have also been shown to play a role in neurological disease. For example, the E4 allele has been associated with increased risk and earlier disease onset in late-onset familial and sporadic Alzheimer's disease [12], faster progression and earlier disease onset in multiple sclerosis [13] and poor prognosis after traumatic brain injury [14–18]. Similar studies in ALS have led to contradictory results. Some have shown no association between any of the APOE alleles and either susceptibility, or the phenotypes age at onset, bulbar onset or survival from symptom onset [19–22], while others have found a relationship between possession of the E4 allele and the phenotypes of bulbar onset [23,24] and shorter survival [25]. Although there have been many studies, the current opinion is that there is no effect of APOE on ALS risk or phenotype, but this cannot be certain. The problem with case-control studies is that they are prone to false positive association if cases and controls are not well matched. This is because differences between the two groups are not due to disease status alone, but also to other hidden attributes. To deal with this problem, proper matching of cases and controls is essential, and one way of doing this is to use family members [26]. The control can either be an unaffected sibling or the non-transmitted parental alleles. One such method is the transmission disequilibrium test [27], which is a joint test of linkage and association performed by examining the probability of transmission of a marker gene from parents to an affected offspring. If this probability is greater than the expectation of 0.5, it can be concluded that the marker is associated with disease. This approach has been used for example, to examine APOE and age of onset as a quantitative trait in ALS, showing that the age of onset of ALS for carriers of the E2 allele is about 3 years later than that for the other alleles [28].

The contradictory nature of the studies into APOE demonstrates the importance of large sample sizes, well matched controls and stringent *p*-values, for reproducible, believable associations. The key to association studies is replication of results, and without this, we should be skeptical about findings. We will now discuss a few other association studies in some detail, but for a full summary, the reader is referred to Table 1.

2.2. SOD1

ALS was first linked to chromosome 21 in 1991 [3] and mutations in SOD1 identified as accounting for 20% of familial cases [2] and 2–7% of sporadic cases [8]. Here we will only discuss its role as it relates to sporadic ALS.

SOD1 is an abundant cytosolic homodimeric protein, and each subunit contains copper and zinc ions in the active site. Its normal function is to reduce the superoxide radical to hydrogen peroxide, which can then be broken down by catalase, preventing oxidative damage. There are two other SOD molecules: SOD2 is a manganese SOD located in mitochondria and SOD3 is an extracellular SOD which also binds copper and zinc. Over 100 mutations in SOD1 have now been discovered, mostly heterozygous missense point mutations [29,30]. Unusually for an enzyme, all mutations are autosomal dominant except for the D90A mutation which can be dominant or

Table 1
A summary of association studies in ALS

Gene	Description	Reason for Investigation	Significance	References
ALAD	D-Aminolevulinic Acid Dehydratase	Lead exposure associated with ALS ALAD is involved in haem synthesis in erythrocytes	No association	[107,108]
ALS2	Alsin	Causes autosomal recessive juvenile ALS (ALS2).	No association	[109–113]
ANG	Angiogenin	ANG is functionally similar to VEGF	Association found in Scottish and Irish populations	[114,115]
APEX	Apurinic endonuclease	Defective DNA repair hypothesis of ALS etiology	May have small role but not major factor	[115]
APOE	Apolipoprotein E	Implicated in several other neurodegenerative disorders	Not associated with susceptibility. May be associated with age of onset, presentation and survival	[19,20,23–25]
AR	Androgen receptor	Causes Kennedy spinal and bulbar muscular atrophy.	No association	[116]
CCS	Copper chaperone for superoxide dismutase	Gene responsible for copper insertion into SOD1	No association	[117]
CNTF	Ciliary neurotrophic factor	Mice lacking CNTF develop mild, progressive motor neuron loss	Contradictory results	[47,49,50,53,118,119]
CYP2D6	Cytochrome p450, subfamily IID, polypeptide 6	Hypothesized poor metabolism of xenobiotics as risk factor	One reported association, not replicable	[120,121]
DCTN1	Dynactin	Disruption of dynein/dynactin complex produces motor neuron disease phenotype in mice	One reported association, not yet replicated	[95]
DNCH1	Dynein heavy chain	Mutations in Dnch1 result in progressive motor neuron degeneration in heterozygous mice, homozygotes also have Lewy-like inclusion bodies,	No association	[90,91,95]
EAAT2	Excitatory amino acid transporter 2	Excitotoxicity hypothesized to result in motor neuron degeneration	No association	[122–128]
HexA	Hexosaminidase A	HexA deficiency causes accumulation of ganglioside GM2 leads to neurodegeneration causing wide spectrum of neurological diseases	Occasionally causes rare ALS-like syndrome	[129]
HFE	Haemochromatosis	Oxidative stress is hypothesized to be implicated in neurodegeneration and misregulation of iron induces oxidative stress. Also, abnormal iron levels found in spinal cords of ALS patients	Contradictory results	[130,131]
LIF	Leukaemia inhibitory factor	LIF is same cytokine family as CNTF, involved in motor neuron survival	One reported association, not yet replicated	[132,133]
LOX	Lysyl oxidase	LOX is a copper containing enzyme and copper-induced cytotoxicity is a hypothetical mechanism of motor neuron degeneration	No association	[134]
MAO-B	Monoamine oxidase B	MAO-B generates free radicals and these are implicated in neuronal damage	Association with age at onset modification reported, not yet replicated	[135]
MAPT	Microtubule-associated protein tau	Microtubule-associated protein *tau* involved in other neurodegenerative diseases and Guam variant of ALS has neurofibrillary tangles containing aggregates of *tau*	Association reported but *p* values were weak	[136,137]
Mito	Mitochondrial DNA deletions	Accumulation of mitochondrial DNA mutations associated with aging development of degenerative diseases. In ALS, abnormal mitochondria are often found in spinal motor neurons	Associations reported, but all studies very small	[138–144]
NAIP	Neuronal apoptosis inhibitory protein	NAIP involved in related disease, spinal muscular atrophy	No association. Mutations in NAIP now considered specific for SMA	[99,101,102,106]
ND2	Subunit 2 of mitochondrial NADH dehydrogenase (Complex I)	Expression of ND2 found in Alzheimer's brains	No association	[145]
NEFH	Neurofilament, heavy chain	Neurofilament accumulation is a hallmark of ALS	Significant association, tail domain deletions present in ~1% ALS patients, not in controls. Findings have replicated in several studies	[78–81,133,146]
PRPH	peripherin	Transgenic mice over-expressing peripherin develop motor neuron degeneration	Few mutations found, not a common cause of ALS	[86,87]
PSEN1	presenilin-1	PSEN1 involved in apoptosis, a postulated mechanism for neuronal death	Weak association found, small study, not replicated	[147]

Table 1 (*continued*)

Gene	Description	Reason for Investigation	Significance	References
PVR	Poliovirus receptor	Poliovirus attacks motor neurons selectively, enteroviral nucleic acids found in spinal cord of ALS patients	Association with lower motor neuron disease found in one small study, not yet replicated	[148]
SETX	Senataxin	Mutations in SETX cause autosomal dominant juvenile ALS (ALS4)	New familial gene, not yet tested in sporadics	[149]
SMN1/2	Survival of motor neuron 1/2	Deletions and mutations of SMN genes cause spinal muscular atrophy	SMN genotypes which reduce SMN protein levels associated with sporadic ALS	[98,104,105,150]
Spastin and paraplegin	Hereditary spastic paraparesis	Mutations in spastin and paraplegin are the most common causes of hereditary spastic paraparesis	One case reported of young onset, slowly progressive upper and lower motor neuron syndrome with spastin mutation. No association with paraplegin	[151,152]
SNCG	Persyn	Persyn is a member of the synuclein family, γ-synuclein. Mutations in α-synuclein found in Parkinson's disease	No association	[153]
SOD1	Cu/Zn superoxide dismutase 1	Mutations in SOD1 account for 20% of familial ALS.	2–7% sporadic cases have mutations	[2,3,5,7,8,29]
SOD2	Manganese superoxide dismutase	SOD2 is a related protein of SOD1, found in mitochondria	No association	[154]
VAPB	Vesicle-associated membrane protein-associated protein B	Mutations in VAPB cause an autosomal dominant, slowly progressive ALS (ALS8)	New familial gene, not yet tested in sporadics	[155]
VDR	Vitamin D receptor	Lead exposure associated with ALS. Vitamin D can affect lead absorption and distribution	Not significant	[107,108]
VEGF	Vascular endothelial growth factor	Susceptibility	Association found in some populations	[59–61,156]

recessive depending upon the genetic background of the individual, and is polymorphic in parts of Scandinavia [31–33]. Mutant SOD1 enzyme activities vary, some being inactive (e.g. H46R) and others near normal (e.g. G93A). There seems to be no correlation between disease onset or duration and mutant SOD1 ability to scavenge superoxide, half-life, solubility, resistance to proteolytic degradation, aggregation potential, or affinity for copper, and the evidence is for a gain of a novel toxic function. How such a diverse range of mutations can result in a novel function producing the same relatively homogeneous phenotype remains a mystery.

2.3. Clinical features of SOD1 ALS

SOD1 ALS is clinically indistinguishable from sporadic ALS. The mean age of onset is consistently 46 to 47 years regardless of the underlying mutation, and in general, there is 90% penetrance by age 70. The mean duration for familial ALS in general is 3–6 years and there is little difference for familial ALS caused by SOD1 mutation. It is not unusual for there to be predominantly lower motor neuron signs, at least to start with. Bulbar onset is unusual and is usually associated with a later onset. There may occasionally be extra-motor symptoms including dementia, autonomic failure, supranuclear extra-ocular muscle weakness and bladder dysfunction.

2.4. Phenotype variation in SOD1 ALS

The phenotype observed in SOD1-mediated familial ALS is highly variable, with age-at-onset, severity and survival all varying wildly, but there is some genotype–phenotype correlation for a few mutations. A4V is the most common ALS-associated SOD1 mutation in North America, accounting for 50% of all mutations [34]. Patients with this mutation have a common phenotype with rapid progression and death occurring at 1.4 years, rather than the 3–5 years which is more typical in other SOD1 mutations [35]. There are other SOD1 mutations with specific phenotype associations, such as I113T which has a much older mean age-at-onset compared with other SOD1 mutations, G37R, which is associated with a significantly earlier age-at-onset and longer survival, L38V, which is associated with earlier age-at-onset and G41D and G93C, which are associated with longer survival [35]. Most mutations do not relate to a single phenotype and the wide range of mutations in SOD1 cannot account for this variability as clinical variation can be observed in members of the same family [29,36]. This suggests that environmental and genetic factors are modifying the phenotype. Many mutations also show incomplete penetrance. The picture is therefore complicated even for the relatively simple situation of a single, large effect, autosomal dominant gene.

The D90A mutation is a case in point. Homozygous affected individuals are more frequent in the Torne Valley, a remote part of northern Sweden and Finland, whereas heterozygous affected are generally from other parts of the world, including southern Sweden, the UK, Belgium and France. The Torne Valley homozygotes have a predictable and relatively benign phenotype, with a generally ascending spastic weakness, long survival and occasional extramotor involvement. Heterozygous affected have a more variable phenotype

which is much more aggressive in line with other SOD1 mutations. The D90A mutation has been shown to have a single founder, about 500 generations ago, with the Torne Valley families becoming isolated approximately 50 generations ago [33,37]. One explanation for these phenotypic differences is that D90A is normally a dominant mutation, consistent with other SOD1 mutations, but that some protective factor tightly linked to SOD1 is found only in the Torne Valley families. Therefore, two copies of the D90A mutation would be required for disease development. Investigation of sequence variants in genes surrounding the SOD1 locus has not found any variants which can account for the differences in phenotype [38,39]. An alternative explanation is that all SOD1 mutations act in an additive fashion to increase the risk of developing ALS. Most mutations would have a large effect size, and so only a small contribution from other genetic or environmental factors would be needed in order to cause disease. This would account for the autosomal dominant inheritance and age-dependant penetrance. If the effect size for D90A was smaller than the other SOD1 mutations, but still more than 50% of the liability threshold, heterozygous individuals would only develop ALS if they also had a number of other risk factors contributing enough to the disease burden. A second copy of the mutation would also be sufficient to cause ALS. This would explain the pattern of recessive inheritance in the Torne Valley families and would also explain the existence of some affected heterozygotes with no family history of ALS. Some families living in particular regions with strong contributory factors would inherit the mutation phenotype as a dominant trait. This has been seen in a French family, which apparently had dominant D90A-mediated ALS [32], but was later shown to have a compound heterozygosity for a second SOD1 mutation [40]. This demonstrates that SOD1 mutations may themselves be part of a general contribution to disease liability.

2.5. Ciliary neurotrophic factor

Ciliary neurotrophic factor (CNTF) was first isolated from chick ciliary ganglia and shown to promote the survival of parasympathetic neurons *in vitro* [41]. It has been shown to have trophic and differentiating effects on many different neural cell types [42–45]. For example, Progressive Motor Neuronopathy (*pmn*) is a naturally occurring autosomal recessive mutation found in mice which resembles spinal muscular atrophy in humans. When mice homozygous for the pmn mutation were treated with CNTF at symptom onset, they showed prolonged survival, improved motor function and fewer of the cellular changes associated with neurodegeneration [46]. Conversely, inactivation of the mouse CNTF gene is not lethal, but the mice develop progressive muscle atrophy, loss of motor neurons and muscle weakness [47], suggesting that in mice at least, CNTF expression is not essential for spinal motor neuron development but is necessary for postnatal neuronal survival.

These results suggest a possible role for CNTF abnormalities in human neurological and psychiatric disorders. Approximately 2% of the human population are homozygous for mutations that inactivate CNTF. So far investigations of such mutations in unipolar affective disorder, schizophrenia and Alzheimer's disease have failed to find any association [48]. Similarly, in ALS, most formal studies of null CNTF mutations have not found association [49–51]. There is however a report of a family with ALS due to SOD1 mutation, one member of which was homozygous null for CNTF and had a much younger onset and more rapid disease course than other affected family members [52]. It was suggested that CNTF was modifying age of onset in this family. This was supported by the observation that mice transgenic for the G93A SOD1 mutation crossed with homozygous null CNTF mice had earlier disease onset than the wild type, but disease duration was unaffected. Although this is a compelling argument, the pattern of SOD1 and CNTF inheritance in this family can equally be explained by chance. A later study of 400 ALS patients found no association between null mutations in CNTF and age of onset, disease progression, survival or clinical presentation [53]. It is therefore difficult to conclude that CNTF is a major modifier of ALS phenotype without further evidence.

2.6. Vascular endothelial growth factor and angiogenin

Vascular endothelial growth factor (VEGF) regulates the growth of new blood vessels and induces vascular permeability. However, VEGF also has additional non-vascular functions. Neuronal axon pathfinding is mediated by semaphorins acting on neuropilin receptors. These receptors are also acted on by various members of the VEGF family [54,55], which implies a role for VEGF in neuronal growth and repair. Additionally, VEGF receptors have been discovered on neurons and astrocytes [56] and on the neural progenitor cells of the retina [57], which suggests that VEGF can act in a neurotrophic and neuroprotective role in the central and peripheral nervous system. Other pro-angiogenic growth factors such as basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) have also been shown to be neurotrophic [58]. It is perhaps not surprising therefore that transgenic mice with a deletion of the hypoxia-response element in the VEGF promoter ($Vegfa^{\delta/\delta}$) develop a progressive motor neuron degeneration at about 5 months of age, reminiscent of ALS [59] (although this was a surprise when first discovered). These mice have reduced levels of VEGF in neural tissue, and induction of VEGF expression by hypoxia in the spinal cord and the brain is diminished.

Following the initial findings in mice, a large multi-centre study was performed to analyse the equivalent region in humans. SNPs and SNP haplotypes were associated with ALS in the Swedish population, Belgian population and a UK population from the Birmingham area. A fourth population from London in the UK did not show an association. In a meta-analysis of the separate centre studies, individuals homozygous for two haplotypes in the VEGF promoter/leader sequence were found to have 1.8 times increased risk of developing ALS [60]. This is one of the largest association studies in ALS to date and included almost 2000 people. It provides good evidence for a true association of VEGF haplotype and susceptibility to ALS,

but there have been criticisms of the statistical interpretation of the findings.

Other studies have been unable to replicate these results [61–64], which may reflect the small sizes of these studies compared to the original study. A possible weak association with affection status and sex has been found [64] and it has been hypothesized that high female to male ratios are needed to detect the association, since this study and the London population in the original study failed to show an association, and they both had low female to male ratios compared to the other populations examined. Further study will have to be done to show whether this is a real association or merely coincidence. Nevertheless, these same haplotypes are associated with a decrease in levels of circulating VEGF and reduction in gene transcription and therefore have a functional effect on the neuronal response to hypoxia. Given the findings in mice, this supports a role in humans, but further studies are required.

A related protein, also involved in the response to hypoxia is angiogenin. This protein is interesting because its gene lies close to that of APEX nuclease which has been implicated in ALS (Table 1), and it is therefore possible that the signal in APEX nuclease is in fact a result of linkage disequilibrium with a true functional variant in angiogenin (ANG). Angiogenin is functionally similar to VEGF, is an inducer of neovascularisation and is required for cell proliferation induced by other angiogenic proteins such as VEGF [65]. A common haplotype associated with a mutation in the angiogenin gene has been identified in patients of Scottish and Irish descent, although an association was not found in other populations studied [65]. Seven missense mutations were identified in both familial and sporadic ALS patients, of which five were within highly conserved residues in catalytic sites of the ANG protein, suggesting a direct effect on the activity of ANG. There is an interaction between ANG and VEGF, in that downregulation of angiogenin reduces VEGF-induced rRNA transcription, and inhibition of nuclear translocation of ANG completely knocks out the angiogenic activity of VEGF. As described above, mice lacking VEGF are known to develop motor neuron degeneration [59] and this provides a possible hypothesis of disease causation for ANG mutations. Interestingly, the effect of ANG mutations seem to be population specific, being found mainly in those with Irish/Scottish ancestry, and it will be important to discover the modifying factors that contribute to disease risk to produce this pattern.

2.7. Neurofilaments and peripherin

One of the characteristic pathological findings in ALS is the presence of accumulations of neurofilaments in the perikarya and proximal axons [66–69]. Neurofilaments are a type of intermediate filament found in neural cells and are thought to play a critical role in supporting the long thin axonal processes. Neurofilaments are classified by size as light (NEFL), medium (NEFM) and heavy (NEFH) chains, and assemble as heteropolymers with a NEFL core with NEFM and NEFH arranged around the outside. Chains are distinguished on the basis of tail length, with NEFM and NEFH containing a repeating lysine–serine–proline (KSP) motif which can be variably phosphorylated. Tail phosphorylation causes extension and electrostatic repulsion and is thought to regulate axonal calibre and interaction with other cytoskeletal components.

Overexpression of the mouse light chain neurofilament gene, NEFL and the human heavy chain neurofilament gene, NEFH, had been shown to cause a neurodegenerative syndrome in transgenic mice [70,71] leading to the hypothesis that overexpression of neurofilaments could be an important part of human disease development. This view was supported by the evidence that disruption of the neurofilament genes to the point where neurofilaments were completely absent protected mice containing the human G85R SOD1 transgene from neurodegeneration [72]. The authors hypothesized that although neurofilaments are important for neuronal survival in development, they contribute to the vulnerability of motor neurons to the toxic effect of G85R SOD1.

When transgenic mice containing mutant SOD1 were crossed with transgenic mice overexpressing the heavy and light chain neurofilament gene [73–75], surprisingly these double transgenic mice had a large improvement in their lifespans and analysis of the spinal cord of 1 year old mice revealed that the massive neurodegeneration typically seen in mutant SOD1 mice was markedly less in double transgenic littermates [73–75]. This confusing picture of neurofilaments in SOD1-mediated ALS suggests that more studies are needed in this area.

Studies of neurofilament mutations in ALS patients have been suggestive of a primary but not major role in ALS. The first of these identified two common variants of the NEFH tail, differing in KSP repeat length, neither variant associated with susceptibility to ALS [76]. A further study by the same group found a codon deletion in five affected individuals and no controls [77]. Two family based studies did not find linkage of the region or mutation in about a hundred patients, suggesting that NEFH variants are not a cause of familial ALS [78,79]. A study of sporadic patients from Scandinavia and the UK identified several mutations in NEFH, accounting for approximately 1% of sporadic ALS cases, and this study also identified a deletion in an individual with familial ALS, although it was not possible to tell if it segregated with disease in this family [80]. Interestingly, all were deletions of multiples of whole repeat motifs, and this pattern also applied to an 84 bp insertion found in a different study [81]. Disease-associated deletions are always paired with the longer wild-type NEFH allele, and the insertion paired with the shorter wild-type NEFH allele. It is possible that the difference in tail lengths is one of the factors increasing susceptibility to ALS, but the picture remains unclear, with the latest study unable to positively confirm the association for NEFH or the other neurofilament subunits [82]. NEFL mutations have been found in a related motor neuron disease, Charcot–Marie–Tooth type 2E [83,84], a hereditary motor and sensory neuropathy.

Peripherin is another type of intermediate filament, preferentially expressed in the peripheral nervous system [85]. A frameshift mutation has been identified in one ALS patient [86], and this was not found in controls. This mutation produced a

truncated protein which when transfected into SW13 cell cultures was unable to self-assemble into neurofilament structures and disrupted NEFL and NEFM assembly into the intermediate filament network. One other mutation has been identified in a patient with sporadic ALS [87]. Immunocytochemistry revealed unusual large aggregates within the spinal motor neurons which contained both neurofilaments and peripherin. Transfected into mice, the mutant protein spontaneously formed aggregates, implying that the mutation was interfering with peripherin assembly.

These studies suggest that genetic variants of intermediate filaments may be a primary cause or contribute significantly towards susceptibility in a small proportion of cases.

2.8. Dynein and dynactin

The neurofilament network is a key part of the axonal transport system, and the pathological findings in ALS taken with the potential primary role of neurofilament mutations, suggest that impairment of axonal transport may be an important step in ALS pathogenesis. In keeping with this, SOD1 mouse models of ALS display impaired retrograde axonal transport [88]. This form of transport is mediated by dyneins, which are microtubule-activated ATPases responsible for transport towards the (−) end of microtubules. They are very large multimeric proteins composed of two or three heavy chains complexed with variable numbers of intermediate and light chains, and require a complex of microtubule-binding proteins that link the cargo to the microtubules. One such complex is dynactin, a well characterised set of at least 8 different subunits, enhancing dynein-dependent motility.

Homozygous loss of the cytoplasmic dynein heavy chain 1 gene (DNCHC1) is often lethal at an early stage of embryogenesis in mice, but heterozygotes are normal [89]. Mutagenesis of this gene has produced two mouse phenotypes with age-related progressive loss of muscle tone and motor ability. Such mutations specifically result in defective fast retrograde transport only observed in alpha motor neurons. Homozygous mice surviving to birth are severely affected and cannot feed or move, dying within 24 h of birth [90]. Lewy-like inclusion bodies and significant loss of spinal anterior horn cells can be observed in homozygotes *in utero*, and these are similar pathological findings to those observed in human ALS and other motor neuron degeneration phenotypes. Despite this, no mutations in human dynein have been found in ALS [91] although mutant SOD1 has been shown to interact with the dynein/dynactin complex [92]. The story for dynactin is however a little different. In *Drosophila*, disrupting the dynactin complex increases the frequency and extent of synaptic retraction events at the neuromuscular junction [93] and this suggests that dynactin is critical for synapse stabilisation. Transgenic mice overexpressing dynamitin, one of the dynactin complex subunits, show inhibited retrograde axonal transport, caused by disruption of the dynein–dynactin complex [94]. These mice develop a late-onset, slowly progressing neurodegeneration similar to human ALS. Dynactin mutations have now been identified in a few familial and sporadic ALS patients [95–97] and it seems likely that genetic variants which impair axonal transport increase susceptibility to ALS.

2.9. SMN genes and copy number variation in complex diseases

Childhood-onset spinal muscular atrophy (SMA) is a disease confined to lower motor neurons and is caused by deletions or mutations in the Survival Motor Neuron (SMN) gene. There are two copies of SMN in humans; SMA is only caused by mutations or deletions in SMN1, but SMN2 copy number is associated with modification of disease severity. Because of the similarities between SMA and ALS, various studies have examined the relationship between the SMN genes and ALS. Studies looking for mutations in SMN1 in ALS patients have not found any association [98–102]. Studies of SMN2 deletion have shown conflicting results [103,104] but these were small studies and it is therefore difficult to draw firm conclusions. Because the chromosome 5 region containing the SMN genes is duplicated, some studies have examined copy number. These have shown that copy number variation that reduces the amount of SMN protein expressed is associated with ALS susceptibility and increased disease severity [98,105]. Further evidence in support of this comes from studies of transgenic SOD1-G86R mice crossbred onto differing genetic backgrounds, which show significantly altered age at onset on some backgrounds compared to others [106]. This effect has been linked to a region of the mouse genome syntenic to human chromosome 5 and which contains the mouse SMN gene.

Both SMN1 and SMN2 contribute to total SMN protein levels. However, SMN2 is less efficient than SMN1 and produces only 20% of SMN transcripts. To assess copy number impact on total SMN protein levels, it is possible to estimate SMN protein level using a formula derived by Veldink and colleagues: SMN protein level = SMN1 copy number + 0.20 × SMN2 copy number [105]. For example, 2 copies of SMN1 and 2 copies of SMN2 would give an SMN level of 2.4. Patients with ALS have been shown to have significantly less total SMN protein than controls, the majority having an SMN level ≤ 2.2, whereas most controls had a level ≥ 2.4.

Copy number variation at the SMN locus may therefore be important in ALS, and copy number in general is being increasingly recognised as a significant cause of genetic disease.

3. Conclusion

The number of replicated association findings in ALS remains depressingly small. No obvious themes of involved proteins have yet emerged, but this remains the hope. Although we continue to make small advances in our understanding of the genetic factors which contribute to disease risk or modify disease phenotype in ALS, there is a great need for large-scale association studies. Despite many years of research and the discovery of a number of genes which cause familial ALS, these still only account for a very small proportion of cases. Candidate gene association studies are limited by their narrow focus on small regions of the

genome, and in a disease like ALS, they can only be informed by uncertain hypotheses of the biological basis of the disease, or by genes already known to cause familial ALS and related diseases. The techniques for genotyping large numbers of genetic markers in a feasible time and at a reasonable cost are now with us. Genome-wide association studies are now a realistic approach for the investigation of the genetic component to sporadic ALS. Recent studies into diseases such as age related macular degeneration, multiple sclerosis, and rheumatoid arthritis show that these sorts of association studies can find robust, replicable associations. These represent our best hope of elucidating mechanisms that underlie the disease and provide new targets for palliative, preventative and curative treatments.

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